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TITLE: Prenatal Exposure to Nicotine and Childhood Asthma: Role of Nicotine Acetylcholine Receptors, Neuropeptides, and Fibronectin Expression in Lung

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14. ABSTRACT We hypothesize that prenatal exposure to nicotine, a major component of tobacco that transverses the placenta, is largely responsible for the development of asthma in children born of mothers who smoke. Specifically, we hypothesize that nicotine is recognized by specific cellular proteins called nicotinic acetylcholine receptors (nAChRs) that are expressed by lung cells termed fibroblasts and pulmonary neuroendocrine cells (PNEC). In fibroblasts, this interaction triggers the exaggerated expression of a connective tissue protein called fibronectin. In PNECs, nicotine stimulates cell growth and the excessive secretion of neuropeptides that affect airway formation and lung growth, and that stimulate smooth muscle cells to contract. In this fashion, nicotine can affect airways development and promote disease during childhood. This proposal will test the hypothesis in animal models of lung development and hyperreactive airways.					
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INTRODUCTION

Asthma is a chronic lung disease characterized by airway dysfunction. Of the many factors implicated in the pathogenesis of asthma, a strong association exists between prenatal and postnatal exposure to environmental tobacco smoke (ETS) (1). This is particularly true in infancy and in childhood where ETS exposure is associated with a higher incidence or prevalence of asthma, and with measures of decreased flow in the airways, bronchial hyperresponsiveness, and increased respiratory infections (2). It has been speculated that the relationship between ETS and asthma is secondary to reduced airway flow caused by tobacco-induced prenatal alterations in airway architecture and/or bronchial reactivity (3,4). However, the exact mechanisms by which prenatal ETS promotes airway dysfunction in children remain unelucidated.

We hypothesize that prenatal exposure to nicotine, a major component of tobacco that transverse the placenta, is largely responsible for childhood asthma in the setting of exposure to ETS. Specifically, we hypothesize that nicotine is recognized by nicotinic acetylcholine receptors (nAChRs) expressed by fibroblasts and pulmonary neuroendocrine cells (PNECs), among other embryonic lung cells. In fibroblasts, this interaction triggers an intracellular signaling cascade that promotes the exaggerated expression and aberrant deposition of fibronectin, a matrix glycoprotein that is highly expressed in developing and injured tissues, and that is found deposited in asthmatic airways (5). The excessive deposition of fibronectin in fetal lungs stimulates cleft formation and the development of an increased number of primitive airway tubules with small caliber in the setting of increased cell proliferation. In PNECs, nicotine stimulates the production of neuropeptides like bombesin (in mice) and gastrin-releasing peptide (GRP; the human counterpart of bombesin) that also stimulate lung branching and cellular proliferation, and that have constrictive effects on bronchial smooth muscle cells. Thus, nicotine induces the excessive deposition of fibronectin and the hyperplasia/hypersecretion of PNECs in developing lungs. These effects are manifested structurally by airway wall remodeling and an increase in the number of small-caliber airways. Functionally, these effects are manifested by airflow limitation and hyperactivity. Together, these events prepare the stage for childhood asthma which is formally established/perpetuated by inflammation induced by continued exposure to ETS and infection in the postnatal period, among other factors. The hypothesis will be tested in specific aims designed to:

- Aim I. Elucidate the mechanisms by which nicotine affects murine lung development using cultured embryonic lung explants.
- Aim II. Examine the effects of prenatal nicotine exposure on postnatal airway structure and function *in vivo*, and study how this relates to fibronectin overexpression and PNEC hyperfunction.

BODY

The following discussion summarizes our recent findings related to Aims I and II:

Distribution of $\alpha 7$ nAChRs in developing murine lungs

We began by exploring the distribution and expression of $\alpha 7$ nAChRs in developing lungs using immunohistochemistry and RT-PCR, respectively. Immunohistochemical studies revealed $\alpha 7$ nAChR protein at all stages of lung development. $\alpha 7$ nAChR protein was identified on both epithelial and mesenchymal cells, particularly around the primitive airways (Figure 1A-

C). However, both immunohistochemistry and RT-PCR analysis suggested that $\alpha 7$ nAChR expression is highest in pseudoglandular stage lungs (embryonic days 11-13) followed by a decrease by day 15 that remains throughout later stages (Figure 1D).

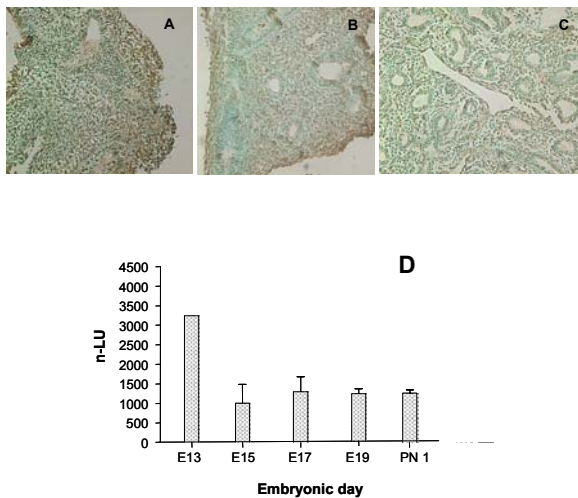
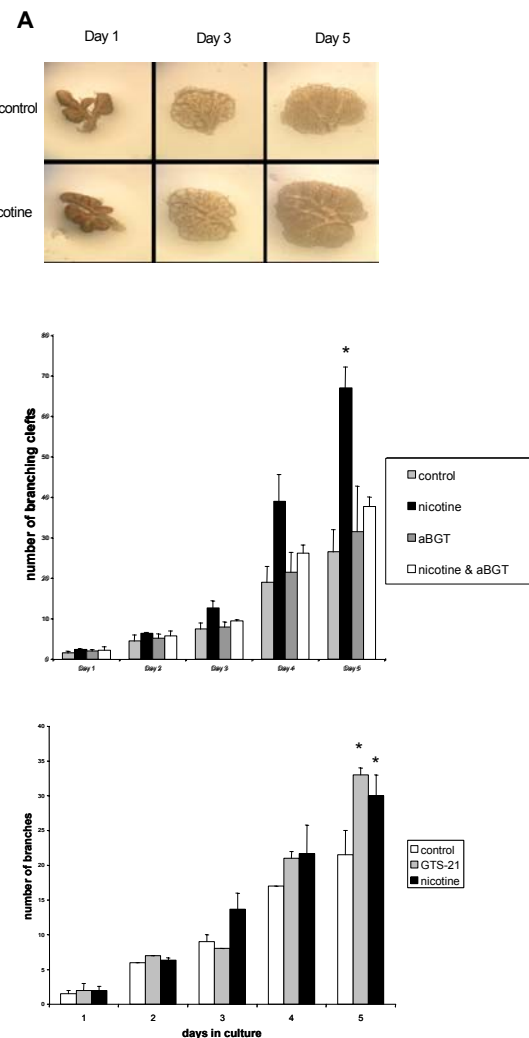


Figure 1. Distribution and expression of $\alpha 7$ nAChRs in the developing murine lung. Murine lungs were harvested at different stages of gestation followed by processing for immunohistochemistry (A-C) or RT-PCR (D) for $\alpha 7$ nAChRs. **A-C, $\alpha 7$ nAChR distribution.** We found abundant staining for $\alpha 7$ nAChRs in the early pseudoglandular stage of lung development (A, E11 lung; B, E13 lung) where expression was detected in both epithelial and mesenchymal cells. Expression of $\alpha 7$ nAChRs diminished at the end of the pseudoglandular stage (C, E15 lung), remaining in the endothelium and epithelium, and continued to decrease as the fetal lung matured into the neonatal period (data not shown). **D, $\alpha 7$ nAChR mRNA expression.** RT-PCR for $\alpha 7$ nAChR showed diminishing mRNA expression between the early and late pseudoglandular stages of development. mRNA expression of $\alpha 7$ nAChR remained at a low level throughout the remainder of gestation (D).

Nicotine stimulates lung branching morphogenesis through $\alpha 7$ nAChRs

To test the effects of nicotine on lung branching morphogenesis, we used the lung explant model; lungs obtained at embryonic day 11 were cultured with varying concentrations of nicotine. In this system, nicotine was found to stimulate branching and this effect was most noticeable at day 4 and 5 of culture (Figure 2, top and middle images). Despite the increased number of branches, the pattern of branching seemed equivalent between groups, and the morphology of the terminal buds was not different. Of note, the nicotine-treated lungs appeared larger than their untreated counterparts suggesting an effect on explant growth (see later).

Figure 2. Nicotine stimulates branching morphogenesis through $\alpha 7$ nAChRs. **Top, Effect of nicotine on ex vivo lung development.** Lungs obtained at embryonic day 11 were placed in culture for 5 days and evaluated daily for branching clefts. Photographs of untreated (controls) and nicotine (1 μ M)-treated lung rudiments were obtained at culture days 1, 3 and 5. **Middle, Effect of nicotine on the branching of lung explants.** Lung rudiments harvested at day 11 were cultured for 5 days in regular media (control) or in the presence of nicotine (1 μ M), α -bungarotoxin (α -BGT, 5 μ M), or both; branching clefts were counted each day. **Bottom, Effect of GTS-21.** Lung rudiments were cultured as described above with nicotine (1 μ M) or GTS-21 (3 μ M) for 5 days and the branching clefts were counted each day.



The stimulatory effect of nicotine on lung branching was entirely inhibited by α -bungarotoxin, a selective antagonist of $\alpha 7$ nAChRs (Figure 2, middle image). Culture with 5 μ M α -bungarotoxin alone in the medium did not impair branching. To further test the role of $\alpha 7$ nAChRs, we treated lung explants with GTS-21, an $\alpha 7$ nAChR agonist also known as DMBX. Like nicotine, GTS-21 stimulated lung branching (Figure 2, lower image).

Nicotine stimulates lung explant growth

To determine the effects of nicotine on lung growth, total DNA content was assessed as a marker of overall explant growth. In the presence of 1 μ M nicotine, the lung explants grew significantly more when compared to controls (Figure 3). Although this effect was diminished by α -bungarotoxin, the antagonist was not as effective in diminishing growth as it was in blocking branching.

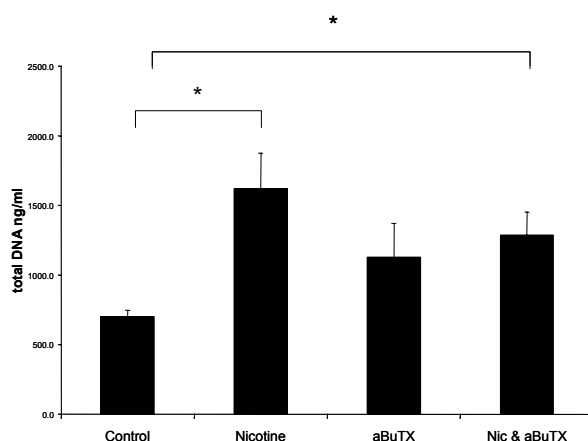


Figure 3. Nicotine stimulates lung growth

Lung rudiments harvested at day 11 of gestation were cultured in the presence of nicotine (1 μ M), α -bungarotoxin (α -BGT, 5 μ M), or both for 5 days. Afterwards, the lungs were collected and processed for DNA fluorometry. Note that nicotine-treated explants contained more DNA, whereas those treated with the combination of nicotine and α -bungarotoxin showed reduced DNA content (* $p < 0.05$).

Other: In addition to the above, we have begun experiments with pulmonary function testing in animals conceived and raised in the setting of nicotine exposure, but no data are available yet. We have also started to examine the distribution of PNECs in lung to address their role in the processes being studied. Finally, we have noticed an increase in fibronectin expression in the walls of vascular structures in the lungs of nicotine-treated animals. This is being investigated further.

KEY RESEARCH ACCOMPLISHMENTS

- Our studies show that nicotine stimulated branching, and that this effect was most evident at culture days 4 and 5. Of note, the dichotomous and monochotomous branching pattern of nicotine-treated lung explants did not differ from that of untreated explants suggesting that nicotine may accelerate branching by an early induction of the same cellular processes involved in normal branching morphogenesis.
- Immunohistochemical and RT-PCR studies confirmed the expression of $\alpha 7$ nAChRs in developing lungs, and showed a relative increase in receptor expression during the pseudoglandular stage of gestation, which coincides with the period of airway development.
- We found that lung branching is inhibited by an antagonist of $\alpha 7$ nAChRs, and is stimulated by an agonist. We also found that the $\alpha 7$ nAChR antagonist reduces branching, but the effect was not as significant.

- The ability of nicotine to affect both lung branching and growth in lung explants suggests that nicotine might cause *dysanaptic lung growth*. Dysanaptic growth refers to the disproportionate growth between conducting airway and alveolar parenchyma first described to explain variability in expiratory flow volume curves. The abnormal lung function associated with prenatal nicotine exposure may be a consequence of dysanaptic growth by changes in branching morphogenesis without an equal change in growth.

REPORTABLE OUTCOMES

- An abstract describing the data presented above has been chosen for oral presentation during a mini-symposium titled: *Best Science at ATS* to be held during the 2006 International American Thoracic Society Meeting in San Diego, CA.
- A manuscript summarizing our data is being prepared for submission for publication.

CONCLUSIONS

Nicotine can affect the development of the primitive airways as well as the growth of the lung. In this fashion, nicotine could promote 'dysanaptic lung growth' which may, in turn, promote airway dysfunction after birth alone or after exposure to inhaled stimulants. Further work is necessary to determine the implications of these events in the clinical setting.

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APPENDICES None